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DESCRIPTION

MARKER Lmx1a SPECIFIC TO DOPAMINE-PRODUCING NEURON

Technical Field

The present invention relates to Lmx1a genes, which are specifically expressed in dopaminergic neurons. Dopaminergic neurons and their progenitor cells are used in transplantation therapy for neurodegenerative diseases such as Parkinson's disease (PD), and can be efficiently isolated by detecting the expression of a Lmx1a gene or a protein encoded by the gene.

Background Art

The dopamine system is an extremely important system for essential regulation of locomotion, hormone secretion, emotions and such in the mammalian brain. Thus, abnormalities in dopaminergic neural transmission cause various neural disorders. For example, Parkinson's disease (PD) is a neurodegenerative disease of the extrapyramidal system that occurs due to specific degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Harrison's Principles of Internal Medicine, Vol. 2, 23rd edition, Isselbacher *et al.*, ed., McGraw-Hill Inc., NY (1994), pp. 2275-7). Oral administration of L-DOPA (3,4-dihydroxyphenylalanine) is performed as a primary therapeutic method for Parkinson's disease to compensate for the decrease in the amount of dopamine produced; however, the duration of the effect is known to be unsatisfactory.

Therapeutic methods have been attempted whereby the midbrain ventral regions of 6- to 9-week old aborted fetuses, which contain dopaminergic neuron progenitor cells, are transplanted to compensate for the loss of dopaminergic neurons (US 5690927; Spencer *et al.* (1992) N. Engl. J. Med. 327: 1541-8; Freed *et al.* (1992) N.Engl. J. Med. 327: 1549-55; Widner *et al.* (1992) N. Engl. J. Med. 327: 1556-63; Kordower *et al.* (1995) N. Engl. J. Med. 332: 1118-24; Defer *et al.* (1996) Brain 119: 41-50; Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-80). However, these methods currently present problems relating to cell supply and ethics (Rosenstain (1995) Exp. Neurol. 33: 106; Turner *et al.* (1993) Neurosurg. 33: 1031-7). Also, various problems are being pointed out, such as the risk of infection and contamination, immunological rejection of transplants (Lopez-Lozano *et al.* (1997) Transp. Proc. 29: 977-980; Widner and Brudin (1988) Brain Res. Rev. 13: 287-324), and low survival rates due to the primary dependence of fetal tissues on lipid metabolism rather than glycolysis (Rosenstein (1995) Exp. Neurol. 33: 106).

In order to resolve ethical issues and the shortage of supply, methods have been

proposed that use, for example, porcine cortex, stria, and midbrain cells (for example, Japanese Patent Kohyo Publication No. (JP-A) H10-508487, JP-A H10-508488 or JP-A H10-509034). In these methods, a complex procedure involving the alteration of cell surface antigens (MHC class I antigens) is required to suppress rejection. Methods involving local immunosuppression by the simultaneous transplantation of Sertoli's cells have been proposed as a method for eliminating transplant rejection (JP-A H11-509170, JP-A H11-501818, Selawry and Cameron (1993) Cell Transplant 2: 123-9). It is possible to obtain transplant cells from relatives with matching MHCs, bone marrow from other individuals, bone marrow banks, or umbilical cord-blood banks. However, if it were possible to use the patient's own cells, the problem of rejection reactions could be overcome without any laborious procedures or trouble.

Therefore, instead of using cells from aborted fetuses as transplant materials, the use of dopaminergic neurons differentiated *in vitro* from non-neural cells such as embryonic stem (ES) cells and bone marrow interstitial cells is considered to be promising. In fact, functional dopaminergic neurons were reported to have been formed upon transplanting ES cells to lesion stria of a rat Parkinson's disease model (Kim *et al.* (2002) Nature 418: 50-56). It is believed that regenerative therapy from ES cells or a patient's own nerve stem cells will be increasingly important in the future.

In the treatment of nerve tissue damage, brain function must be reconstructed, and in order to form suitable links with surrounding cells (network formation), it is necessary to transplant immature cells, which can differentiate *in vivo* into neurons. When transplanting neuron progenitor cells, in addition to the aforementioned problem regarding supply, there is also the possibility that the progenitor cells will differentiate into groups of heterogeneous cells. For example, in treating Parkinson's disease, it is necessary to selectively transplant catecholamine-containing neurons that produce dopamine. Examples of transplant cells proposed in the past for use in the treatment of Parkinson's disease include striatum (Lindvall *et al.* (1989) Arch. Neurol. 46: 615-31; Widner *et al.* (1992) N. Engl. J. Med. 327: 1556-63), immortalized cell lines derived from human fetal neurons (JP-A H08-509215; JP-A H11-506930; JP-A No. 2002-522070), human postmitotic neurons derived from NT2Z cells (JP-A H09-5050554), primordial neuron cells (JP-A H11-509729), cells and bone marrow stroma cells transfected with exogenous genes so as to produce catecholamines such as dopamines (JP-A 2002-504503; JP-A 2002-513545), and genetically engineered ES cells (Kim *et al.* (2002) Nature 418: 50-56). However, none of these contain only dopaminergic neurons or cells that differentiate into dopaminergic cells.

A method has been proposed for selectively concentrating and isolating dopaminergic neurons from undifferentiated cell populations. In this method, a reporter gene expressing a fluorescent protein is introduced into each cell of a cell population, under the control of a gene

promoter/enhancer, such as the tyrosine hydroxylase (TH) expressed in dopaminergic neurons. Fluorescing cells are then isolated. The dopaminergic neurons are visualized in their viable state, and concentrated, isolated, and identified (Japanese Patent Application Kokai Publication No. (JP-A) 2002-51775 (unexamined, published Japanese patent application)). This method requires the complicated step of introducing an exogenous gene. Further, the presence of a reporter gene poses problems of toxicity and immunogenicity when used in conjunction with gene therapy.

Lmx1a was identified as a LIM-type homeobox gene expressed in the roof plate of the developing spinal cord (the organizer region which secretes differentiation-inducing factors in the most dorsal region; neurons do not develop from this region), the neural crest, the hindbrain rhombic lip, and the posterior region of the developing cerebral hemisphere (Non-Patent Documents 1 and 2). In dreher mutant mice, which are animal models for type II agyria, autosomal recessive mutations have been revealed to occur on Lmx1a (Non-Patent Document 1). Furthermore, it is also known that mutations in Lmx1a trigger lesions in the central nervous system in queue courte (qc) rats (Non-Patent Documents 3 to 5). The Lmx1a gene is expressed not only during the fetal phase but also after birth, and contributes to the formation of the cerebral cortex and the cerebellum.

Non-Patent Document 1: Millonig *et al.* (2000) Nature 403: 764-769

Non-Patent Document 2: Failli *et al.* (2002) Mechanisms of Development 118(1-2): 225-228

Non-Patent Document 3: Kitada *et al.* (2001) 2001 Meeting on Physiological Genomics and Rat Models

Non-Patent Document 4: Kitada *et al.* (2001) The 15th International Mouse Genome Conference

Non-Patent Document 5: Kitada *et al.* (2000) Record of the 17th Meeting of the Japanese Society of Animal Models for Human Diseases

Disclosure of the Invention

Problems to be Solved by the Invention

One of the major current problems in Parkinson's disease (PD) transplantation therapy is that dopaminergic neuron progenitor cells which are induced to differentiate, as well as the midbrain ventral region of aborted fetuses, are both mixtures of a myriad of cell types. When considering safety in neural circuit formation, it is preferable to use isolated cells that comprise only the cell type of interest. Furthermore, when considering the risk of tumorigenesis, it is believed preferable to use isolated postmitotic neurons. Moreover, when considering the survival of cells at their transplantation site in the brain, and their ability to properly form networks, it is expected that therapeutic effects can be further improved by isolating progenitor cells at the earliest stage possible. Therefore, the inventors of the present invention aimed to

isolate genes specific to dopaminergic neuron progenitor cells. As a result, they successfully isolated Lrp4 (WO 2004/065599) and the novel gene 65B13 (WO 2004/038018), which are genes transiently expressed in neuron progenitor cells immediately before and after cell cycle exit.

Means to Solve the Problems

In order to isolate genes specific to dopaminergic neuron progenitor cells, a gene specifically expressed in the most ventral region of the E12.5 murine midbrain containing dopaminergic neurons was identified using a modification ("Method for Homogenizing the Amounts of DNA Fragments and Subtraction Method", WO 02/103007) of the subtraction method (N-RDA: Representational Difference Analysis; RDA (Listsyn N.A. (1995) Trends Genet. 11: 303-7), by additionally dividing the ventral region into two regions in the dorsoventral direction. One of the isolated fragments was a cDNA fragment encoding Lmx1a. Hitherto, Lmx1a has been reported as being expressed in the hippocampus, the cerebellum, and the most dorsal side of the roof plate region covering the diencephalon to the spinal cord. However, Lmx1a expression was not known to be specific to dopaminergic neurons.

Lmx1a expression was maintained from the stages of proliferating progenitor cells to cells after cell cycle exit and even in adults. In this way, Lmx1a showed an expression pattern that differed from known markers. In particular, it possesses the characteristics of genes expressed from the stage of proliferating progenitor cells. For this reason, it was considered to be a useful marker for detecting dopaminergic neurons including proliferating progenitor cells. Also, by its combined use with known markers, Lmx1a enabled sorting of proliferating progenitor cells and postmitotic precursor cells. Thus in this way the present invention was expected to be an effective marker, particularly when screening for differentiation-inducing reagents for dopaminergic neurons. Further, Lmx1a is a marker expressed at an earlier stage than known markers, and this also contributes to the above expectation.

Specifically, the present invention provides:

[1] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the step of contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to a transcript of a gene that consists of a nucleotide sequence of any one of (1) to (6):

(1) the nucleotide sequence of SEQ ID NO: 13;

(2) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14;

(3) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the

nucleotide sequence of SEQ ID NO: 13;
(4) the nucleotide sequence of SEQ ID NO: 15 or 17;
(5) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 16 or 18; and
(6) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17;

[2] the method of [1], wherein the polynucleotide comprises at least 15 nucleotides;

[3] a reagent for distinguishing a dopaminergic neuron and/or a progenitor cell thereof, wherein the reagent comprises, as an active ingredient, a polynucleotide that hybridizes under stringent conditions to a transcript of a gene that consists of a nucleotide sequence of any one of (1) to (6):
(1) the nucleotide sequence of SEQ ID NO: 13;
(2) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14;
(3) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;
(4) the nucleotide sequence of SEQ ID NO: 15 or 17;
(5) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 16 or 18; and
(6) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17;

[4] the reagent of [3], wherein the polynucleotide comprises at least 15 nucleotides;

[5] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the step of contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with an antibody that binds to a polypeptide that consists of an amino acid sequence of any one of (1) to (6) or a partial sequence thereof:
(1) the amino acid sequence of SEQ ID NO: 14;
(2) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 14;
(3) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;
(4) the amino acid sequence of SEQ ID NO: 16 or 18;
(5) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 16 or 18; and
(6) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent

conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17;

[6] the method of [5], wherein the polypeptide consisting of a partial sequence comprises at least six consecutive amino acid residues;

[7] a reagent for distinguishing a dopaminergic neuron and/or a progenitor cell thereof wherein the reagent comprises, as an active ingredient, an antibody that binds to a polypeptide that consists of an amino acid sequence of any one of (1) to (6) or a partial sequence thereof:

(1) the amino acid sequence of SEQ ID NO: 14;

(2) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 14;

(3) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(4) the amino acid sequence of SEQ ID NO: 16 or 18;

(5) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 16 or 18; and

(6) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17;

[8] the reagent of [7], wherein the polypeptide consisting of a partial sequence comprises at least six consecutive amino acid residues;

[9] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the steps of:

(a) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to a transcript of a gene that consists of a nucleotide sequence from any one of (a-1) to (a-6):

(a-1) the nucleotide sequence of SEQ ID NO: 13;

(a-2) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14;

(a-3) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(a-4) the nucleotide sequence of SEQ ID NO: 15 or 17;

(a-5) the nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 16 or 18; and

(a-6) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and

(b) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3,

and TH, or with an antibody that binds to translation products of said selected genes;

[10] the method of [9], which further comprises the step of:

(c) contacting a cellular sample potentially comprising the dopaminergic neuron and/or the progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of either or both of the genes selected from DAT and ADH2 or with an antibody that binds to a translation product of a said selected gene;

[11] the method of [9], wherein the gene selected in step (b) is one or more of Lmx1b, Nurr1, or En1;

[12] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the steps of:

(a) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to a transcript of a gene that consists of a nucleotide sequence from any one of (a-1) to (a-6):

(a-1) the nucleotide sequence of SEQ ID NO: 13;

(a-2) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14;

(a-3) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(a-4) the nucleotide sequence of SEQ ID NO: 15 or 17;

(a-5) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence (human) of SEQ ID NO: 16 or 18; and

(a-6) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and

(b) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of either or both of the genes selected from DAT and ADH2, or with an antibody that binds to translation products of said selected genes;

[13] the method of any one of [9] to [12], wherein the polynucleotide is a nucleotide sequence comprising at least 15 consecutive nucleotides;

[14] a kit for distinguishing a dopaminergic neuron and/or a progenitor cell thereof, wherein the kit comprises: the reagent of [3] or [4]; and a polynucleotide that hybridizes under stringent conditions to transcripts of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT, and ADH2;

[15] a kit for distinguishing a dopaminergic neuron and/or a progenitor cell thereof, wherein the kit comprises: the reagent of [3] or [4]; and an antibody that binds to translation products of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT,

and ADH2;

[16] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the steps of:

(a) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with an antibody that binds to a polypeptide consisting of an amino acid sequence of any one of (a-1) to (a-6) or a partial sequence thereof:

(a-1) the amino acid sequence of SEQ ID NO: 14;

(a-2) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 14;

(a-3) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(a-4) the amino acid sequence of SEQ ID NO: 16 or 18;

(a-5) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 16 or 18; and

(a-6) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and

(b) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, and TH, or with an antibody that binds to translation products of said selected genes;

[17] the method of [16], which further comprises the step of:

(c) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of either or both of the genes selected from DAT and ADH2 or with an antibody that binds to translation products of said selected genes;

[18] the method of [16], wherein the genes selected in step (b) is one or more of Lmx1b, Nurr1, or En1;

[19] a method for detecting or selecting a dopaminergic neuron and/or a progenitor cell thereof, wherein the method comprises the steps of:

(a) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with an antibody that binds to a polypeptide consisting of an amino acid sequence of any one of (a-1) to (a-6) or a partial sequence thereof:

(a-1) the amino acid sequence of SEQ ID NO: 14;

(a-2) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 14;

(a-3) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(a-4) the amino acid sequence of SEQ ID NO: 16 or 18;

(a-5) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 16 or 18; and

(a-6) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and

(b) contacting a cellular sample potentially comprising a dopaminergic neuron and/or a progenitor cell thereof with a polynucleotide that hybridizes under stringent conditions to transcripts of either or both of the genes selected from DAT and ADH2, or with an antibody that binds to translation products of said selected genes;

[20] the method of any one of [16] to [19], wherein the polypeptide consisting of a partial sequence comprises at least 6 consecutive amino acid residues;

[21] a kit for distinguishing a dopaminergic neuron and/or progenitor cell thereof, wherein the kit comprises: the reagent of [7] or [8]; and a polynucleotide that hybridizes under stringent conditions to transcripts of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT, and ADH2;

[22] a kit for distinguishing a dopaminergic neuron and/or a progenitor cell thereof, wherein the kit comprises: the reagent of [7] or [8]; and an antibody that binds to translation products of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT, and ADH2;

[23] a method of screening for a differentiation-inducing reagent for a dopaminergic neuron, wherein the method comprises the steps of:

(a) contacting a test substance with cells that can be differentiated into dopaminergic neurons;

(b) detecting a transcript of the Lmx1a gene by contacting the cells, after contact with the test substance, with a polynucleotide that hybridizes under stringent conditions to a transcript of a gene consisting of a nucleotide sequence of any one of (b-1) to (b-6):

(b-1) the nucleotide sequence of SEQ ID NO: 13;

(b-2) a nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14;

(b-3) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;

(b-4) the nucleotide sequence of SEQ ID NO: 15 or 17;

(b-5) the nucleotide sequence encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 16 or 18; and

(b-6) a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and
(c) determining whether the test substance can induce the differentiation of dopaminergic neurons when the transcript of the Lmx1a gene is detected;

[24] the method of [23], wherein the polynucleotide is a nucleotide sequence comprising at least 15 consecutive nucleotides;

[25] a method of screening for a differentiation-inducing reagent for a dopaminergic neuron, wherein the method comprises the steps of:
(a) contacting a test substance with cells that can be differentiated into dopaminergic neurons;
(b) detecting a translation product of the Lmx1a gene by contacting the cells, after contact with the test substance, with an antibody that binds to a polypeptide consisting of an amino acid sequence of any one of (b-1) to (b-6) or a partial sequence thereof:
(b-1) the amino acid sequence of SEQ ID NO: 14;
(b-2) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 14;
(b-3) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 13;
(b-4) the amino acid sequence of SEQ ID NO: 16 or 18;
(b-5) an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of SEQ ID NO: 16 or 18; and
(b-6) an amino acid sequence encoded by a nucleotide sequence that hybridizes under stringent conditions to a gene consisting of the nucleotide sequence of SEQ ID NO: 15 or 17; and
(c) determining whether the test substance can induce the differentiation of dopaminergic neurons when the translation product of the Lmx1a gene is detected; and

[26] the method of [25], wherein the polypeptide consisting of a partial sequence comprises at least six consecutive amino acid residues.

Brief Description of the Drawings

Fig. 1 is a set of photographs showing the expression of the dopaminergic neuron marker gene tyrosine hydroxylase (TH) in the ventral region of an E12.5 mouse midbrain. The left-hand photograph is a sagittal section and the right-hand photograph is a coronal section performed along the line in the left-hand photograph. Furthermore, the midbrain region was cut into four regions, and VL from subtracted from V. Mes: mesal, D: dorsal, DL: dorsal lateral, VL: ventral lateral, and V: ventral.

Fig. 2 is a set of photographs showing the results of analysis of Lmx1a, Lmx1b, Nurr1, and tyrosine hydroxylase (TH) mRNA expression in the midbrain of an E12.5 mouse, using *in*

situ hybridization.

Fig. 3 is a set of photographs showing the results of coexpressing Lmx1a and tyrosine hydroxylase (TH) proteins in the midbrain of an E12.5 mouse, as detected by immunostaining.

Fig. 4 is a set of photographs showing the results of coexpressing Lmx1a and En1 proteins in the midbrain of an E12.5 mouse, as detected by immunostaining.

Fig. 5 is a set of photographs showing the results of Lmx1a expression and BrdU incorporation in the midbrain of an E12.5 mouse, as detected by immunostaining. Lmx1a was shown to be expressed in proliferating dopamine neuron progenitor cells.

Fig. 6 is a set of photographs showing the results of Lmx1a and DAT mRNA expression in the midbrain of a mouse seven days after birth, as detected by *in situ* hybridization. The arrow indicates the substantia nigra region of the midbrain.

Fig. 7 is a set of photographs showing the results of Lmx1a expression in the human adult brain region, as detected by RT-PCR. SN: substantia nigra, Pu: putamen, CC: cerebral cortex, Ce: cerebellum, Hi: hippocampus, MO: medulla oblongata, CN: caudate nucleus, and Po: pons.

Fig. 8 schematically shows the timing of expression of Lmx1a and the dopaminergic neuron-specific marker genes. The abscissa shows the time of development and maturation of dopaminergic neurons. Lmx1a was considered to be expressed in dopaminergic neurons at all stages of differentiation.

Best Mode for Carrying Out the Invention

<Marker Polynucleotide Probes>

The "dopaminergic neuron marker polynucleotide probes" of the present invention are used as markers that select and/or detect dopaminergic neuron progenitor cells before and after proliferation, and dopaminergic neurons. These polynucleotides can detect the expression of Lmx1a gene, which is expressed at all stages of differentiation, from dopaminergic neuron progenitor cells before cell cycle exit to dopaminergic neurons in adults. The nucleotide sequences of Lmx1a genes are known. For example, the sequence of mouse Lmx1a can be referred to in Nature 403: 764-769 (2000); Nature 420: 563-573 (2002); Mech. Dev. 118: 225-228 (2002); and such, and is also registered in GenBank under the Accession No. NM_033652. Furthermore, human Lmx1a has been reported in Gene 290: 217-225 (2002). The genomic sequence of human Lmx1a is registered in GenBank under the Accession No. AH011517, and isoforms such as the 6A mutant (GenBank Accession No. NM_177398) have also been reported.

Here, a "marker polynucleotide probe" refers to a polymer composed of a number of nucleotides, such as deoxyribonucleic acids (DNAs) or ribonucleic acids (RNAs), or nucleotide pairs that should be able to detect expression of the Lmx1a gene. Double-stranded cDNA can

also be used as a probe in tissue *in situ* hybridization, and such double-stranded cDNAs are included in the markers of the present invention. RNA probes (riboprobes) are particularly preferable as marker polynucleotide probes for detecting RNA in tissues. When detecting Lmx1a gene expression by using the presence or absence of mRNA expression, it is preferable to use the region encoding Lmx1a. The amino acid sequence of mouse Lmx1a is registered in GenBank as NP_387501.1. The coding region corresponds to nucleotides 220 to 1368 in the sequence of NM_033652, and its specific sequence is shown in SEQ ID NO: 14. In addition, the amino acid sequence of human Lmx1a is also known (GenBank Accession No. AAL82892.1, etc.), and its specific sequences are shown in SEQ ID NOs: 16 and 18.

If needed, the marker polynucleotide probes of the present invention can also contain non-naturally-occurring nucleotides such as 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, dihydrouridine, 2'-O-methylpseudouridine, β-D-galactosylqueuosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N6-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, β-D-mannosylqueuosine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine, 5-methoxyuridine, 2-methylthio-N6-isopentenyladenosine, N-((9-β-D-ribofuranosyl-2- methylthiopurin-6-yl)carbamoyl)threonine, N-((9-β-D- ribofuranosylpurin-6-yl)N-methylcarbamoyl)threonine, uridine-5-oxyacetic acid-methyl ester, uridine-5-oxyacetic acid, wybutoxosine, pseudouridine, queuosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 4-thiouridine, 5-methyluridine, N-((9-β-D-ribofuranosylpurin-6-yl)carbamoyl)threonine, 2'-O-methyl-5-methyluridine, 2'-O-methyluridine, wybutosine, and 3-(3-amino-3-carboxy propyl)uridine.

The marker polynucleotide probes of the present invention comprise nucleotide sequences that are complementary to the mRNA of Lmx1a, which is specifically expressed in dopaminergic neurons. For example, the nucleotide sequence of mouse Lmx1a cDNA is registered in GenBank with the Accession No. NM_033652, and its specific sequence is shown in SEQ ID NO: 13. Polynucleotides with a sequence complementary to this cDNA can be used as the marker polynucleotide probes of the present invention. Furthermore, the amino acid sequence of mouse Lmx1a is registered in GenBank under the Accession No. NP_387501.1 (SEQ ID NO: 14); however, due to degeneration of the genetic code, its nucleotide sequence may differ from the nucleotide sequence of above-mentioned GenBank Accession No. NM_033652 (SEQ ID NO: 13). Polynucleotides complementary to such degenerate sequences are also comprised in the marker polynucleotide probes of the present invention. Furthermore, in

addition to degenerate sequences, polynucleotides complementary to nucleotide sequences that hybridize under stringent conditions to Lmx1a genes (genes comprising the nucleotide sequence of SEQ ID NO: 13) are also comprised in the marker polynucleotide probes that can be used in the present invention.

Similarly, human Lmx1a sequences are also known (SEQ ID NOs: 15 and 17), and the marker polynucleotide probes of the present invention also comprise polynucleotides with a sequence complementary to human Lmx1a mRNA, polynucleotides with a degenerate relationship to these polynucleotides, and polynucleotides complementary to nucleotide sequences that hybridize under stringent conditions to human Lmx1a genes (SEQ ID NOs: 15 and 17).

Herein, the phrase "complementary to a nucleotide sequence" comprises not only nucleotide sequences that completely match a template, but also those in which at least 70%, preferably 80%, more preferably 90%, and even more preferably 95% or more (for example, 97%, 98% or 99%) of the nucleotides of the sequence match the template. It is known that genes with such high homology are likely to encode polypeptides with the same function. Herein, "matching" refers to the formation of a chain in which: adenine (A) in the nucleotide sequence of the template polynucleotide pairs with thymine (T) (or urasil (U) in case of RNA), T or U pair with A, cytosine (C) pairs with (G), and G pairs with C . Homology between specific nucleotide chains at the nucleotide sequence level can be determined, for example, by the BLAST algorithm (Altschul (1990) Proc. Natl. Acad. Sci. USA 90: 5873-7). BLASTN (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-410) has been developed as a program for determining homology between nucleotide sequences based on this algorithm, and can be used to determine the homology between a marker polynucleotide probe sequence and a template sequence. For specific analysis methods refer to http://www.ncbi.nlm.nih.gov, for example.

Alternative isoforms and allelic variants may exist for Lmx1a. Polynucleotides that can detect such isoforms and allelic variants can also be used as marker polynucleotide probes of the present invention. In addition, as mentioned above, there is a high possibility that genes with high homology will encode polypeptides with the same function. Furthermore, such polynucleotides having high homology can often form pairs under stringent hybridization conditions. Therefore, the marker polynucleotide probes of the present invention comprise polynucleotides that comprise nucleotide sequences that hybridize under stringent conditions to a polynucleotide consisting of the nucleotide sequence encoding Lmx1a, which is specifically expressed in dopaminergic neurons. Such isoforms, allelic mutants, and polynucleotides having high homology can be obtained from cDNA libraries and genomic libraries of animals such as humans, mice, rats, rabbits hamsters, chickens, pigs, cows, goats, and sheep by, for example, preparing probes based on known nucleotide sequences of the Lmx1a gene, and using these

probes to carry out known hybridization methods such as colony hybridization, plaque hybridization, and Southern blotting. See "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)) for methods of constructing cDNA libraries. In addition, commercially available cDNA libraries or genomic libraries may also be used.

Construction of a cDNA library is explained in more detail below: first, total RNA is prepared from cells, organs, tissues or such that express Lmx1a, using known techniques such as guanidine ultracentrifugation (Chirwin *et al.* (1979) Biochemistry 18: 5294-5299) or AGPC (Chomczynski and Sacchi (1987) Anal. Biochem. 162: 156-159). Then, an mRNA Purification Kit (Pharmacia) or such is used to purify mRNAs from the total RNA prepared. Alternatively, mRNAs can also be obtained using a kit for direct mRNA preparation, such as a QuickPrep mRNA Purification Kit (Pharmacia). Next, cDNAs are synthesized from the resulting mRNAs using reverse transcriptase. cDNA synthesis kits, such as the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation), are also commercially available. Other methods that use the 5'-RACE method to synthesize and amplify cDNAs by PCR may also be used (Frohman *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 8998-9002; Belyavsky *et al.* (1989) Nucleic Acids Res. 17: 2919-32). In addition, in order to construct cDNA libraries containing a high percentage of full-length clones, known techniques such as the oligo-capping method (Maruyama and Sugano (1994) Gene 138: 171-4; Suzuki (1997) Gene 200: 149-56) can also be employed. The cDNAs obtained in this manner are then incorporated into suitable vectors.

Examples of hybridization conditions suitable for use in the present invention include "2x SSC, 0.1% SDS, 50°C", "2x SSC, 0.1% SDS, 42°C", and "1x SSC, 0.1% SDS, 37°C". Conditions of higher stringency include "2x SSC, 0.1% SDS, 65°C", "0.5x SSC, 0.1% SDS, 42°C", and "0.2x SSC, 0.1% SDS, 65°C". More specifically, a method that uses the Rapid-hyb buffer (Amersham Life Science) can be carried out by performing pre-hybridization at 68°C for 30 minutes or more, adding a probe to allow hybrid formation at 68°C for one hour or more, washing three times in 2x SSC/0.1% SDS at room temperature for 20 minutes per wash, washing three times in 1x SSC/0.1% SDS at 37°C for 20 minutes per wash, and finally washing twice in 1x SSC/0.1% SDS at 50°C for 20 minutes per wash. This can also be carried out using, for example, the Expresshyb Hybridization Solution (CLONTECH), by performing pre-hybridization at 55°C for 30 minutes or more, then adding a labeled probe and incubating at 37°C to 55°C for one hour or more, washing three times in 2x SSC/ 0.1% SDS at room temperature for 20 minutes per wash, and washing once at 37°C for 20 minutes with 1x SSC/0.1% SDS. Herein, conditions of higher stringency can be achieved by setting a high temperature for pre-hybridization, hybridization, and the second wash. For example, a pre-hybridization and hybridization temperature of 60°C can be raised to 68°C for higher stringency. In addition to factors such as the salt concentration of the buffer and temperature, one with

ordinary skill in the art can also integrate other factors, such as probe concentration, probe length, nucleotide sequence composition of the probe, and reaction time, to obtain Lmx1a isoforms and allelic variants, and corresponding genes derived from other organisms.

For detailed information on hybridization procedures refer to Molecular Cloning, A Laboratory Manual 2nd ed. (Cold Spring Harbor Press (1989); Section 9.47-9.58), Current Protocols in Molecular Biology (John Wiley & Sons (1987-1997); Section 6.3-6.4), DNA Cloning 1: Core Techniques, A Practical Approach 2nd ed. (Oxford University (1995); Section 2.10 for conditions, in particular). Examples of hybridizing polynucleotides include polynucleotides containing a nucleotide sequence that has at least 50% or more, preferably 70% or more, more preferably 80% or more and even more preferably 90% or more (for example, 95% or more, or furthermore 96%, 97%, 98%, or 99% or more) identity with a known nucleotide sequence of Lmx1a. Such identities can be determined using the BLAST algorithm (Altschul *et al.* (1990) Proc. Natl. Acad. Sci. USA 87: 2264-8; Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-7) as for determining homology. In addition to the above-described BLASTN program for nucleotide sequences, the BLASTX program for determining the identity of amino acid sequences (Altschul *et al.* (1990) J. Mol. Biol. 215: 403-10) has also been developed based on this algorithm and can be used to determine identity at the amino acid level. As described above, http://www.ncbi.nlm.nih.gov can be referred to for specific examples of analysis methods.

Genes with an Lmx1a-like structure and function, such as Lmx1a isoforms and allelic variants, can be obtained from cDNA libraries and genomic libraries of animals such as mice, rats, rabbits, hamsters, chickens, pigs, cows, goats, sheep, and primates comprising humans by designing primers based on known nucleotide sequences of the Lmx1a gene, using gene amplification technology (PCR) (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Sections 6.1-6.4).

The polynucleotide sequences can be confirmed by using conventional methods for determining sequence. For example, the dideoxynucleotide chain termination method (Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74: 5463) can be used. In addition, sequences can also be analyzed using a suitable DNA sequencer.

Moreover, since it is preferable that the marker polynucleotide probes of the present invention should specifically detect Lmx1a expression, the present marker polynucleotide probes comprise polynucleotides that consist of nucleotide sequences containing at least 15 consecutive nucleotides from each of the nucleotide sequences of: (1) a sequence complementary to Lmx1a mRNA; (2) a sequence complementary to the nucleotide sequence encoding the amino acid sequence of Lmx1a; and (3) a sequence hybridizing under stringent conditions to Lmx1a mRNA. Such polynucleotides comprising nucleotide sequences that contain at least 15 consecutive

nucleotides can be used as probes for detecting Lmx1a mRNA expression, or as PCR or RT-PCR primers for amplifying Lmx1a mRNA. When used as probes the polynucleotides normally consist of 15 to 100, and preferably 15 to 35 nucleotides, and when used as primers they normally consist of at least 15 or more and preferably around 30 nucleotides. When using as primers, the polynucleotides can be designed to have a restriction enzyme recognition sequence or tag or such added to their 5'-end, and at their 3' end, a sequence complementary to a target sequence. Such polynucleotides, which consist of nucleotide sequences comprising at least 15 consecutive nucleotides, can hybridize with Lmx1a mRNAs.

Marker polynucleotide probes of the present invention can be prepared from cells that express Lmx1a using the aforementioned hybridization or PCR or such. In addition, they can also be produced by chemical synthesis based on known Lmx1a sequence data. Riboprobes, which are considered to be particularly preferable for detecting RNAs in tissue, can be obtained by inserting a cloned Lmx1a gene or portion thereof into plasmid vector pSP64 in the reverse direction, followed by run-off transcription of the inserted sequence portion. Although pSP64 contains an SP6 promoter, methods for producing riboprobes by combining phage T3, T7 promoter and RNA polymerase are also known.

<Antibodies>

The present invention provides anti-Lmx1a antibodies that can be used for tissue immunostaining and such of dopaminergic neurons. Antibodies of the present invention include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, single-chain antibodies (scFv) (Huston *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 5879-83; The Pharmacology of Monoclonal Antibody, Vol. 113, Rosenburg and Moore ed., Springer Verlag (1994) pp. 269-315), humanized antibodies, multispecific antibodies (LeDoussal *et al.* (1992) Int. J. Cancer Suppl. 7: 58-62; Paulus (1985) Behring Inst. Mitt. 78: 118-32; Millstein and Cuello (1983) Nature 305: 537-9; Zimmermann (1986) Rev. Physiol. Biochem. Pharmacol. 105: 176-260; VanDijk *et al.* (1989) Int. J. Cancer 43: 944-9), and antibody fragments such as Fab, Fab', $F(ab')_2$, Fc, and Fv. Moreover, the antibodies of the present invention may also be modified by PEG and such, as necessary. Antibodies of the present invention can also be produced in the form of fusion proteins with β-galactosidase, maltose-binding protein, GST, green fluorescent protein (GFP) and such, to allow detection without the use of a secondary antibody. In addition, the antibodies can be modified by labeling with biotin or such, to allow detection or recovery using avidin, streptavidin, or such.

The antibodies of the present invention are specific to any of: (1) a polypeptide encoded by a Lmx1a gene (SEQ ID NOs: 13, 15, and 17); (2) a Lmx1a polypeptide (SEQ ID NOs: 14, 16, and 18); (3) a polypeptide comprising an amino acid sequence with a deletion, insertion,

substitution, or addition of one or more amino acids in a Lmx1a polypeptide (SEQ ID NOs: 14, 16, and 18); (4) a polypeptide encoded by a nucleotide sequence that hybridizes under stringent conditions to a sequence complementary to the nucleotide sequenc of a Lmx1a gene (SEQ ID NOs: 13, 15, and 17); and (5) a partial fragment from any of the above-mentioned polypeptides of (1) to (4), which has antibody-inducing antigenicity, such as a polypeptide comprising at least six amino acid residues (for example 6, 8, 10, 12 or 15 amino acid residues or more).

The antibodies of the present invention can be produced by using, as a sensitizing antigen, an Lmx1a polypeptide or fragment thereof, or cells expressing the same. In addition, short fragments of Lmx1a polypeptide may also be used as immunogens when coupled to a carrier such as bovine serum albumin, Keyhole-limpet hemocyanin, and ovalbumin. In addition, Lmx1a polypeptides or fragments thereof may be used in combination with known adjuvants, such as aluminum adjuvant, Freund's complete (or incomplete) adjuvant, or pertussis adjuvant, to enhance the immune response to the antigen.

The "Lmx1a polypeptides" for obtaining the antibodies of the present invention comprise naturally occurring Lmx1a derived from humans, mice, and such. The amino acid residues constituting the Lmx1a polypeptide may be naturally occurring or modified amino acids. Moreover, the Lmx1a polypeptides of the present application comprise fusion proteins modified by other peptide sequences.

The Lmx1a polypeptides for obtaining the antibodies of the present invention should have the antigenicity of Lmx1a and comprise a polypeptide comprising an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acid residues in the amino acid sequence of naturally occurring Lmx1a. It is well known that mutant polypeptides consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids, maintain the same biological activity as the original polypeptide (Mark *et al.* (1984) Proc. Natl. Acad. Sci. USA 81: 5662-6; Zoller and Smith (1982) Nucleic Acids Res. 10: 6487-500; Wang *et al.* (1984) Science 224: 1431-3; Dalbadie-McFarland *et al.* (1982) Proc. Natl. Acad. Sci. USA 79: 6409-13). Methods for deleting, inserting, substituting, or adding one or more amino acids in a given protein while maintaining the original antigenicity of the protein are known in the art. For example, polynucleotides encoding mutant proteins may be prepared by site-directed mutagenesis and expressed appropriately to obtain the mutant proteins (Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989); Current Protocols in Molecular Biology, John Wiley & Sons, (1987-1997), Section 8.1-8.5; Hashimoto-Goto *et al.* (1995) Gene 152: 271-5; Kunkel (1985) Proc. Natl. Acad. Sci. USA 82: 488-92; Kramer and Fritz (1987) Method. Enzymol. 154: 350-67; Kunkel (1988) Method. Enzymol. 85: 2763-6).

The antibodies of the present invention comprise those that are specific to a portion of

an Lmx1a polypeptide. In other words, as well as polypeptides with the full-length amino acid sequence of Lmx1a, the "Lmx1a polypeptides" for obtaining the antibodies of the present invention comprise polypeptide fragments with sequences that are identical to at least six amino acid residues or more (for example, 6, 8, 10, 12 or 15 amino acid residues or more) of Lmx1a. Particularly preferable fragments can be exemplified by a polypeptide fragment of the Lmx1a amino terminus, carboxyl terminus, or such . The Lmx1a polypeptide fragments include fragments containing an α-helix and α-helix forming region, an α-amphipathic region, a β-sheet and β-sheet forming region, a β-amphipathic region, a substrate binding region, a high antigen index region, a coil and coil forming region, a hydrophilic region, a hydrophobic region, a turn and turn forming region, and a surface forming region. In the context of the present application, Lmx1a polypeptide fragments may be any fragments, as long as they have the antigenicity of an Lmx1a polypeptide. The antigen-determining site of a polypeptide can be predicted using methods for analyzing the hydrophobicity/hydrophilicity of the amino acid sequence of a protein (Kyte-Doolittle (1982) J. Mol. Biol. 157: 105-22), or methods of secondary structure analysis (Chou-Fasman (1978) Ann. Rev. Biochem. 47: 251-76), and can be confirmed using computer programs (Anal. Biochem. 151: 540-6 (1985)) or the PEPSCAN method in which a short peptide is synthesized before its antigenicity is confirmed (Japanese Patent Kohyo Publication No. (JP-A) S60-500684 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication)), or the like.

Lmx1a polypeptides and Lmx1a polypeptide fragments can be isolated from Lmx1a-expressing cells, tissues, or organs, based on their physical properties. Alternatively, these polypeptides and polypeptide fragments can also be produced using known genetic recombination techniques, chemical synthesis methods, or such. For example, for *in vitro* Lmx1a polypeptide production, Lmx1a polypeptides can be produced in an *in vitro* cell-free system using *in vitro* translation methods (Dasso and Jackson (1989) Nucleic Acids Res. 17: 3129-44). In contrast, when producing polypeptides using cells, a polynucleotide that encodes a polypeptide of interest is first incorporated into an appropriate expression vector, a suitable cell host is selected, and then the cells are transformed by the expression vector. The polypeptide of interest can then be obtained by culturing the transformed host cells under conditions that express the polypeptide most efficiently.

Vectors for expressing polypeptides include vectors of various origins, such as plasmids, cosmids, viruses, and bacteriophages (Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989); Current Protocols in Molecular Biology, John Wiley & Sons (1987)). Vectors comprise regulatory sequences into which a desired polynucleotide is incorporated so it will be under the control of the regulatory sequences when expressed in transfected host cells. Here, the "regulatory sequences" include promoters, ribosome binding sites and terminators in

the case of a prokaryotic host cells, and promoters and terminators in the case of eukaryotic host cells, and in some cases the sequences may also contain transactivators, transcription factors, poly A signals which stabilize transcription products, splicing and polyadenylation signals, and others. Such regulatory sequences comprise all the components required for the expression of polynucleotides linked thereto. The vectors may further comprise selection markers. Alternatively, a signal peptide necessary to transfer an intracellularly expressed polypeptide into the lumen of the endoplasmic reticulum or outside of the cell, or, when the host is a Gram negative microbe, into the periplasm or extracellular space, can also be incorporated into an expression vector such that its nucleotide sequence is linked to the polypeptide of interest and expressed. Various signal peptides derived from heterogeneous proteins are known and can be used. Linkers may also be added, and start (ATG) or stop codons (TAA, TAG, or TGA) may be inserted as necessary.

Examples of vectors capable of expressing polypeptides *in vitro* include pBEST (Promega). In addition, various vectors are known to be suitable for expression in prokaryotic hosts (see, *e.g.*, "Basic Microbiology Course 8 - Genetic Engineering" (Kyoritsu Publishing)). One with ordinary skill in the art can suitably select vectors suitable for the hosts and suitable methods for introducing vectors into the hosts. Various host cells are known. For example, fungal cells such as yeasts and molds, higher plants, insects, fish, amphibians, reptiles, birds, mammalian cells and such, and various culture cell systems (COS, HeLa, C127, 3T3, BHK, HEK293, Bowes melanoma cells, myeloma, Vero, Namalwa, Namalwa KJM-1, and HBT5637 (Japanese Patent Application Kokai Publication No. (JP-A) S63-299 (unexamined, published Japanese patent application)), etc.) can also be used as hosts to express Lmx1a polypeptides and their antigenic fragments. Vector systems suitable for each cell and methods for introducing a vector into host cells are also known. Moreover, methods for expressing exogenous proteins in animals *in vivo* (see, *e.g.*, Susumu (1985) Nature 315: 592-4; Lubon (1998) Biotechnol. Annu. Rev. 4: 1-54) and in plant bodies are also known, and can be used to express Lmx1a polynucleotides.

DNAs can be inserted into vectors by ligase reactions using restriction enzyme sites (Current Protocols in Molecular Biology, John Wiley & Sons (1987) Section 11.4-11.11; Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1989) Section 5.61-5.63). In addition, the Lmx1a polypeptide expression vectors can be designed as necessary by selecting nucleotide sequences with high expression efficiency in view of the frequency of the host's codon usage (Grantham *et al.* (1981) Nucleic Acids Res. 9: r43-74). The cells of the hosts that produce the Lmx1a polypeptides comprise polynucleotides encoding the Lmx1a polypeptides. So long as the polynucleotides are expressed properly, the polynucleotides themselves may be regulated by their own promoter, incorporated in the host

genome, or maintained as extrachromosomal structures.

The host cells are cultured using known methods appropriate to the host cells selected. For example, when animal cells are the hosts, the cells can be cultured for about 15 to 200 hours at a pH of about 6 to 8 and a temperature of 30°C to 40°C, using a medium such as DMEM (Virology 8: 396 (1959)), MEM (Science 122: 501 (1952)), RPMI1640 (J. Am. Med. Assoc. 199: 519 (1967)), 199 (Proc. Soc. Biol. Med. 73: 1 (1950)), or IMDM, adding serum such as fetal calf serum (FCS), as necessary. In addition, the medium may be replaced, aerated, or stirred during the course of culturing, as necessary.

Normally, the Lmx1a polypeptides produced by gene recombination techniques can be recovered from the medium if they are secreted outside of the cells or from body fluids when the host is a transgenic organism. When the polypeptides are produced inside the cells, the cells are dissolved and the polypeptides are recovered from the dissolution products. The polypeptides of interest are then purified by suitably combining known methods of protein purification, such as salting out, distillation, various types of chromatography, gel electrophoresis, gel filtration, ultrafiltration, recrystallization, acid extraction, dialysis, immunoprecipitation, solvent precipitation, solvent extraction, and ammonium sulfate or ethanol precipitation. Examples of chromatography include ion exchange chromatography, such as anion or cation exchange chromatography, affinity chromatography, reversed-phase chromatography, adsorption chromatography, gel filtration chromatography, hydrophobic chromatography, hydroxyapatite chromatography, phosphocellulose chromatography, and lectin chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Marshak *et al.* ed., Cold Spring Harbor Laboratory Press (1996)). The proteins can be purified by liquid phase chromatography, such as HPLC or FPLC, using various types of columns. In addition, for example, when a desired protein is expressed as a fusion protein with GST, purification methods using glutathione columns are effective. On the other hand, proteins with histidine tags can be purified using nickel columns. When the Lmx1a polypeptides are produced as fusion proteins, they can be purified and then unnecessary portions can be removed using enzymes such as thrombin or factor Xa, as necessary.

Naturally occurring polypeptides can also be purified based on their physical properties and used as antigens to obtain the antibodies of the present invention. The purified polypeptides can also be modified using enzymes such as chymotrypsin, glucosidase, trypsin, protein kinase, and lysyl endopeptidase, as necessary. In addition to the aforementioned chemical synthesis and genetic engineering techniques used for the Lmx1a polypeptides, Lmx1a polypeptide fragments can also be produced by using suitable enzymes, such as peptidases, to cleave an Lmx1a polypeptide.

Polyclonal antibodies specific to dopaminergic neurons can be obtained from, for

example, the serum collected from immunized animals after immunizing mammals with purified Lmx1a polypeptides or fragments thereof, coupled to adjuvants as required. Although there are no particular limitations as to the mammals used, typical examples include rodents, lagomorphs and primates. Specific examples include rodents such as mice, rats and hamsters, lagomorphs such as rabbits, and primates such as monkeys, including cynomolgus monkeys, rhesus monkeys, baboons and chimpanzees. Animals can be immunized by suitably diluting and suspending a sensitizing antigen in phosphate-buffered saline (PBS) or physiological saline, mixing with an adjuvant as necessary until emulsified, and injecting into an animal intraperitoneally or subcutaneously. The sensitizing antigens mixed with Freund's incomplete adjuvant are preferably administered several times every four to 21 days. Antibody production can be confirmed by using conventional methods to measure the level of an antibody of interest in the serum. Finally, the serum itself may be used as a polyclonal antibody, or it may be further purified. See, for example, "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Sections 11.12-11.13) for specific methods.

Monoclonal antibodies can be produced by removing the spleen from an animal immunized in a manner described above, separating immunocytes from the spleen, and fusing them with a suitable myeloma cell using polyethylene glycol (PEG) or such to establish hybridomas. Cell fusion can be carried out according to the Milstein method (Galfre and Milstein (1981) Methods Enzymol. 73: 3-46). Cells that allow chemical selection of fused cells are particularly preferable for the myeloma cells. When using such myeloma cells that allow chemical selection, fused hybridomas can be selected by culturing in a culture medium (HAT culture medium) containing hypoxanthine, aminopterin, and thymidine, which destroys non-fused cells. Next, clones that produce antibodies against Lmx1a polypeptides, or fragment thereof, are selected from the established hybridomas. The selected clones are then introduced into the abdominal cavities of mice or such, and ascites is collected to obtain the monoclonal antibodies. For information on specific methods see "Current Protocols in Molecular Biology" (John Wiley & Sons (1987) Section 11.4-11.11).

Hybridomas can also be obtained by first using an immunogen to sensitize human lymphocytes that have been infected by EB virus *in vitro*, then fusing the sensitized lymphocytes with human myeloma cells (such as U266) to obtain hybridomas that produce human antibodies (Japanese Patent Application Kokai Publication No. (JP-A) S63-17688 (unexamined, published Japanese patent application)). In addition, human antibodies can also be obtained by using antibody-producing cells generated by sensitizing transgenic animals which have the repertoire of human antibody genes (WO92/03918; WO93/02227; WO94/02602; WO94/25585;; WO96/34096; Mendez *et al.* (1997) Nat. Genet. 15: 146-156, etc.). Methods that do not use hybridomas can be exemplified by methods in which cancer genes are introduced to immortalize

immunocytes, such as antibody-producing lymphocytes.

In addition, antibodies can also be produced by genetic recombination techniques (see Borrebaeck and Larrick (1990) Therapeutic Monoclonal Antibodies, MacMillan Publishers Ltd., UK). First, a gene that encodes an antibody is cloned from hybridomas or antibody-producing cells (such as sensitized lymphocytes). The resulting gene is then inserted into a suitable vector, the vector is introduced into a host, and the host is cultured to produce the antibody. This type of recombinant antibody is also included in the antibodies of the present invention. Typical examples of recombinant antibodies include chimeric antibodies, which comprise a non-human antibody-derived variable region and a human antibody-derived constant region, and humanized antibodies, which comprise a non-human-derived antibody complementarity determining region (CDR), human antibody-derived framework region (FR), and human antibody constant region (Jones *et al.* (1986) Nature 321: 522-5; Reichmann *et al.* (1988) Nature 332: 323-9; Presta (1992) Curr. Op. Struct. Biol. 2: 593-6; Methods Enzymol. 203: 99-121 (1991)).

Antibody fragments can be produced by treating the aforementioned polyclonal or monoclonal antibodies with enzymes such as papain or pepsin. Alternatively, an antibody fragment can be produced by genetic engineering techniques using a gene that encodes an antibody fragment (see Co *et al.*, (1994) J. Immunol. 152: 2968-76; Better and Horwitz (1989) Methods Enzymol. 178: 476-96; Pluckthun and Skerra (1989) Methods Enzymol. 178: 497-515; Lamoyi (1986) Methods Enzymol. 121: 652-63; Rousseaux *et al.* (1986) 121: 663-9; Bird and Walker (1991) Trends Biotechnol. 9: 132-7).

Multispecific antibodies include bispecific antibodies (BsAb), diabodies (Db), and such. Multispecific antibodies can be produced by methods such as (1) chemically coupling antibodies having different specificities with different types of bifunctional linkers (Paulus (1985) Behring Inst. Mitt. 78: 118-32), (2) fusing hybridomas that secrete different monoclonal antibodies (Millstein and Cuello (1983) Nature 305: 537-9), or (3) transfecting eukaryotic cell expression systems, such as mouse myeloma cells, with a light chain gene and a heavy chain gene of different monoclonal antibodies (four types of DNA), followed by the isolation of a bispecific monovalent portion (Zimmermann (1986) Rev. Physio. Biochem. Pharmacol. 105: 176-260; Van Dijk *et al.* (1989) Int. J. Cancer 43: 944-9). On the other hand, diabodies are dimer antibody fragments comprising two bivalent polypeptide chains that are constructed by gene fusion. These can be produced using known methods (see Holliger *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 6444-8; EP404097; WO93/11161).

Antibodies and antibody fragments can be recovered and purified using Protein A and Protein G. They can also be purified by the protein purification techniques described above, in the same way as for non-antibody polypeptides (Antibodies: A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)). For example, when using Protein A

to purify an antibody of the present invention, known Protein A columns such as Hyper D, POROS, or Sepharose F.F. (Pharmacia) can be used. The concentration of the resulting antibody can be determined by measuring absorbance or by using an enzyme linked immunoadsorbent assay (ELISA).

The antigen binding activity of an antibody can be determined by measuring absorbance, or by using fluorescent antibody methods, enzyme immunoassay (EIA) methods, radioimmunoassay (RIA) methods, or ELISA. When ELISA is used, an antibody of the present invention is first immobilized onto a support, such as a plate. An Lmx1a polypeptide is then added, and a sample containing the antibody of interest is added. Herein, the samples containing an antibody of interest include, for example, culture supernatants of antibody-producing cells, purified antibodies, and such. Next, a secondary antibody that recognizes an antibody of the present invention is added, and the plate is incubated. The plate is then washed and the label attached to the secondary antibody is detected. Specifically, if a secondary antibody is labeled with alkaline phosphatase, for example, its antigen binding activity can be determined by adding an enzyme substrate such as p-nitrophenyl phosphate, and then measuring the absorbance. In addition, a commercially available system such as BIAcore (Pharmacia) can also be used to evaluate antibody activities.

<Detection of Dopaminergic Neurons>

The present invention provides methods for selectively detecting dopaminergic neurons. Dopaminergic neurons at all stages of differentiation, from proliferating dopaminergic neuron progenitor cells before cell cycle exit to mature dopaminergic neurons, can be detected using Lmx1a expression as an index. The methods for detecting dopaminergic neurons using polynucleotides or antibodies of the present invention can be used to diagnose disorders, such as Parkinson's disease, that involve degeneration of dopaminergic neurons. Dopaminergic neurons can be detected using the marker polynucleotide probes or antibodies of the present invention.

More specifically, the present invention provides methods for detecting dopaminergic neurons, comprising the step of contacting a marker polynucleotide probe of the present invention with a cell sample containing potential dopaminergic neurons. In these methods, the marker polynucleotide probes are preferably labeled with a radioactive isotope or non-radioactive compound. Examples of radioactive isotopes used for labeling include ^{35}S and ^{3}H. When using a radiolabeled marker polynucleotide probe, RNAs that bind to the marker can be detected by using emulsion autoradiography to detect silver particles. In addition, commonly known non-radioactive isotopes for labeling a marker polynucleotide probe include biotin and digoxigenin. A biotin-labeled marker can be detected using, for example, fluorescently labeled

avidin, or avidin labeled with an enzyme such as alkaline phosphatase or horseradish peroxidase. Digoxigenin-labeled markers can be detected using anti-digoxigenin antibodies labeled with fluorescence or an enzyme, such as alkaline phosphatase or horseradish peroxidase. When using enzyme labeling, detection is carried out by incubating with the enzyme substrate to form a stable pigment at the marker location. Fluorescent *in situ* hybridization (FISH) is a convenient and particularly preferable method for detecting dopaminergic neurons.

Furthermore, the present invention provides methods for detecting dopaminergic neurons, which comprise the step of contacting an anti-Lmx1a antibody with a cellular sample potentially comprising dopaminergic neurons. Specifically, cells expressing an Lmx1a polypeptide and dopaminergic neurons at all differentiation stages, from mature dopaminergic neurons to progenitor cells before proliferation, can be detected and selected by contacting cellular samples predicted to comprise dopaminergic neurons or progenitor cells thereof with an antibody of the present invention, and selecting the cells that bind to the antibody. To simplify this detection/selection, the antibodies of the present invention can be labeled or immobilized onto a solid phase. For detection, techniques such as ELISA, RIA, and surface plasmon resonance may be combined. When purification of the selected dopaminergic neurons or progenitor cells thereof is required, the antibodies of the present invention may be used in affinity chromatography.

Also, by combining the markers of the present invention with conventional markers that detect dopaminergic neurons or progenitor cells thereof, it becomes possible to sort between progenitor cells and mature neurons, and further to sort between progenitor cells before and after division.

For example, progenitor cells and mature neurons can be sorted by combining Lmx1a with DAT and/or ADH2 as markers. As mentioned above, the Lmx1a gene is confirmed to be widely expressed during differentiation from progenitor cells before division to mature dopaminergic neurons. On the other hand, as shown in the Examples described below, the DAT and ADH2 genes are expressed after the cells have differentiated into dopaminergic neurons. Therefore, progenitor cells and mature neurons can be separately detected or selected by detecting the expression of an Lmx1a gene using a marker polynucleotide probe or antibody of the present invention and further analyzing the expression of either the DAT gene, the ADH2 gene, or both in cells in which Lmx1a gene expression was detected.

The first method of the present invention for detecting or selecting dopaminergic neurons and/or progenitor cells thereof comprises the steps of:

(a) contacting a polynucleotide that can hybridize under stringent conditions to an Lmx1a gene transcript with a cellular sample that potentially comprises dopaminergic neurons and/or progenitor cells thereof; and

(b) contacting a polynucleotide that hybridizes under stringent conditions to transcripts of one gene selected from the DAT gene or ADH2 gene, or both, or contacting an antibody that binds to the translation products of this selected gene with a cellular sample potentially comprising dopaminergic neurons and/or progenitor cells thereof.

The second method of the present invention for detecting or selecting dopaminergic neurons and/or progenitor cells thereof comprises the steps of:

(a) contacting an antibody that binds to a polypeptide comprising the amino acid sequence of Lmx1a or a partial sequence thereof with a cellular sample potentially comprising dopaminergic neurons and/or progenitor cells thereof; and

(b) contacting a polynucleotide that hybridizes under stringent conditions to transcripts of one gene selected from the DAT gene or ADH2 gene, or both, or contacting an antibody that binds to the translation products of this selected gene with a cellular sample potentially comprising dopaminergic neurons and/or progenitor cells thereof.

In both the first and second methods mentioned above, Lmx1a gene expression is analyzed in step (a). To detect Lmx1a gene expression at the transcriptional level, as in the first method, a "polynucleotide that can hybridize under stringent conditions to a Lmx1a gene transcript" is used. The aforementioned marker polynucleotide probes of the present invention can be used as this polynucleotide. To detect Lmx1a gene expression at the translational level, as in the second method, an "antibody that binds to a polypeptide comprising an amino acid sequence of Lmx1a or a partial sequence thereof" is used. The aforementioned anti-Lmx1a antibodies of the present invention can be used as this antibody. In this way, both methods analyze Lmx1a expression in step (a).

In step (b), to investigate whether cells are differentiated mature dopaminergic neurons, the expression of the DAT gene and/or ADH2 gene is examined based on their transcripts or translation products.

Polynucleotides that can detect DAT mRNAs are used as the detection polynucleotides for detecting DAT gene expression based on transcripts. Such polynucleotides for DAT detection, which can hybridize to a DAT mRNA, comprise the following: (1) DNAs or RNAs comprising a nucleotide sequence complementary to a DAT cDNA (SEQ ID NOs: 39 and 41); (2) DNAs or RNAs comprising a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 40 or 42 and which is a degenerate sequence of the DAT gene code; and (3) DNAs or RNAs comprising a nucleotide sequence that can hybridize under stringent conditions to the nucleotide sequence of SEQ ID NO: 39 or 41. On the other hand, DAT-binding antibodies are used to detect DAT gene expression at the translational level. DAT-binding antibodies comprise antibodies that are specific to any one of: (1) a DAT polypeptide (SEQ ID NOs: 40 and 42); (2) a polypeptide comprising an amino acid

sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of a DAT polypeptide (SEQ ID NOs: 40 and 42) ; and (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Similarly polynucleotides that can detect ADH2 mRNAs are used as the detection polynucleotides for detecting ADH2 gene expression based on transcripts. Such polynucleotides for ADH2 detection, which can hybridize to an ADH2 mRNA, comprise the following: (1) DNAs or RNAs comprising a nucleotide sequence complementary to an ADH2 cDNA (SEQ ID NOs: 43 and 45); (2) DNAs or RNAs comprising a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 44 or 46 and which is a degenerate sequence of the DAT gene code; and (3) DNAs or RNAs comprising a nucleotide sequence that can hybridize under stringent conditions to an ADH2 cDNA (SEQ ID NOs: 43 and 45). On the other hand, ADH2-binding antibodies are used to detect ADH2 gene expression at the translational level. ADH2-binding antibodies comprise antibodies specific to any one of: (1) an ADH2 polypeptide (SEQ ID NOs: 44 and 46); (2) a polypeptide comprising an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of an ADH2 polypeptide (SEQ ID NOs: 44 and 46) ; or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

As described above, progenitor cells can be selectively detected or selected by subtracting the group of cells in which DAT gene and/or ADH2 gene expression was detected from the group of cells in which Lmx1a gene expression was detected. The detection of expression of Lmx1a gene and the DAT and/or ADH2 genes can be conducted at the same time or in succession. When conducted at the same time, as an example, the expression of each gene can be detected simultaneously by adding different tags to each detection probe or such. When conducted in succession, cells in which Lmx1a gene expression is detected can be selected first, and the presence or absence of DAT and/or ADH2 gene expression can then be confirmed. Alternatively, a group of cells expressing the Lmx1a gene can be selected from a group of cells in which DAT and/or ADH2 gene expressions were not detected.

Next, detection or selection of groups of cells before or after division among the progenitor cells can be achieved by combining as markers, (a) the Lmx1a gene and (b) one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, and TH. Lmx1b, Nurr1, En1, Ptx3, and TH are a group of genes expressed in postmitotic precursor cells. Therefore, the expression of these genes can be used to distinguish progenitor cells after cell cycle exit. On the other hand, the Lmx1a gene is also expressed in proliferating progenitor cells before cell cycle exit. Thus, proliferating progenitor cells before cell cycle exit can be detected or selected from among dopaminergic neuron progenitor cells by detecting or selecting

cells expressing Lmx1a but do not express Lmx1b, Nurr1, En1, Ptx3, or TH.

Specifically, the first method of the present invention for detecting or selecting dopaminergic neuron progenitor cells by distinguishing progenitor cells before and after cell cycle exit comprises the steps of:

(a) contacting a polynucleotide that can hybridize under stringent conditions to a Lmx1a gene transcript with a cellular sample potentially comprising dopaminergic neuron progenitor cells; and

(b) contacting a polynucleotide that hybridizes under stringent conditions to a transcript of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, and TH; or contacting an antibody that binds to a translation product of these genes with a cellular sample potentially comprising dopaminergic neuron progenitor cells.

The second method of the present invention for detecting or selecting dopaminergic neuron progenitor cells by distinguishing progenitor cells before and after cell cycle exit comprises the steps of:

(a) contacting an antibody that binds to the translation product of an Lmx1a gene with a cellular sample potentially comprising dopaminergic neuron progenitor cells; and

(b) contacting a polynucleotide that hybridizes under stringent conditions to a transcript of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, and TH; or contacting an antibody that binds to a translation product of these genes with a cellular sample potentially comprising dopaminergic neuron progenitor cells.

In both the first and second methods mentioned above, Lmx1a gene expression is detected in step (a) based on transcripts or translation products, as in the aforementioned "method for selecting progenitor cells using Lmx1a and DAT or such". The method and materials for detecting Lmx1a gene expression are the same as those of the aforementioned "method for selecting progenitor cells using Lmx1a and DAT or such".

In step (b), the expression of one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, and TH is analyzed based on transcripts or translation products to distinguish postmitotic precursor cells from among the dopaminergic neuron progenitor cells.

Polynucleotides that can detect Lmx1b mRNAs are used as detection polynucleotides for detecting "Lmx1b gene" expression at the transcriptional level. Such polynucleotides for Lmx1b detection can hybridize to Lmx1b mRNAs and comprise the following : (1) DNAs or RNAs comprising a nucleotide sequence complementary to a Lmx1b cDNA (SEQ ID NOs: 19 and 21); (2) DNAs or RNAs comprising a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 20 or 22 and which is a degenerate sequence of Lmx1b gene code; and (3) DNAs or RNAs consisting of a nucleotide sequence that can hybridize under stringent conditions to the nucleotide sequence of SEQ ID NO: 19 or 21.

When detecting Lmx1b gene expression at the translational level, Lmx1b-binding antibodies are used. Lmx1b-binding antibodies comprise antibodies that are specific to anyone of: (1) an Lmx1b polypeptide (SEQ ID NOs: 20 and 22); (2) a polypeptide consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of an Lmx1b polypeptide (SEQ ID NOs: 20 and 22); or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Polynucleotides that can detect Nurr1mRNAs are used as detection polynucleotides for detecting "Nurr1 gene" expression at the transcriptional level. Such polynucleotides for Nurr1 detection can hybridize to Nurr1mRNAs and comprise the following: (1) DNAs or RNAs comprising a nucleotide sequence complementary to a Nurr1 cDNA (SEQ ID NOs: 23 and 25); (2) DNAs or RNAs consisting of a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 24 or 26 and which is a degenerate sequence of Nurr1 gene code; and (3) DNAs or RNAs consisting of a nucleotide sequence that can hybridize under stringent conditions to an Nurr1 cDNA (SEQ ID NOs: 23 and 25). When detecting Nurr1gene expression at the translational level, Nurr1-binding antibodies are used. Nurr1-binding antibodies comprise antibodies that are specific to anyone of: (1) a Nurr1 polypeptide (SEQ ID NOs: 24 and 26); (2) a polypeptide consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of a Nurr1 polypeptide (SEQ ID NOs: 24 and 26); or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Polynucleotides that can detect En1mRNA are used as detection polynucleotides for detecting "En1 gene" expression at the transcriptional level. Such polynucleotides for En1 detection can hybridize to En1mRNAs and comprise the following: (1) DNAs or RNAs consisting of a nucleotide sequence complementary to an En1 cDNA (SEQ ID NOs: 27 and 29); (2) DNAs or RNAs consisting of a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 28 or 30 and which is a degenerate sequence of En1 gene code; and (3) DNAs or RNAs consisting of a nucleotide sequence that can hybridize under stringent conditions to an En1 cDNA (SEQ ID NOs: 27 and 29). When detecting En1 gene expression at the translational level, En1-binding antibodies are used. En1-binding antibodies comprise antibodies that are specific to anyone of: (1) an En1 polypeptide (SEQ ID NOs: 28 and 30); (2) a polypeptide consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of an En1 polypeptide (SEQ ID NOs: 28 and 30); or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Polynucleotides that can detect Ptx3mRNAs are used as detection polynucleotides for detecting "Ptx3 gene" expression at the transcriptional level. Such polynucleotides for Ptx3

detection can hybridize to Ptx3mRNAs and comprise the following: (1) DNAs or RNAs consisting of a nucleotide sequence complementary to an Ptx3 cDNA (SEQ ID NOs: 31 and 33); (2) DNAs or RNAs consisting of a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 32 or 34 and which is a degenerate sequence of Ptx3 gene code; and (3) DNAs or RNAs consisting of a nucleotide sequence that can hybridize under stringent conditions to a Ptx3 cDNA (SEQ ID NOs: 31 and 33). When detecting ADH2 gene expression at the translational level, Ptx3-binding antibodies are used. Ptx3-binding antibodies comprise antibodies that are specific to anyone of: (1) a Ptx3 polypeptide (SEQ ID NOs: 32 and 34); (2) a polypeptide consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of a Ptx3 polypeptide (SEQ ID NOs: 32 and 34); or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Polynucleotides that can detect TH mRNAs are used as detection polynucleotides for detecting "TH gene" expression at the transcriptional level. Such polynucleotides for TH detection can hybridize to TH mRNAs and comprise the following: (1) DNAs or RNAs consisting of a nucleotide sequence complementary to a TH cDNA (SEQ ID NOs: 35 and 37); (2) DNAs or RNAs consisting of a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 36 or 38 and which is a degenerate sequence of TH gene code; and (3) DNAs or RNAs consisting of a nucleotide sequence that can hybridize under stringent conditions to a TH cDNA (SEQ ID NOs: 35 and 37). When detecting TH gene expression at the translational level, TH-binding antibodies are used. TH-binding antibodies comprise antibodies that are specific to anyone of: (1) a TH polypeptide (SEQ ID NOs: 36 and 38); (2) a polypeptide consisting of an amino acid sequence with a deletion, insertion, substitution, or addition of one or more amino acids in the amino acid sequence of a TH polypeptide (SEQ ID NOs: 36 and 38); or (3) a polypeptide fragment comprising at least six amino acid residues from the polypeptide of (1) or (2).

Of these marker genes that can be used in step (b), selection using Lmx1b, Nurr1, or En1 as the marker is preferable. Expression of these three genes can be detected immediately after cell cycle exit, thus enabling detection and selection with a more accurate distinction of progenitor cells before and after cell cycle exit.

As mentioned above, by subtracting a group of cells in which expression of a Lmx1b, Nurr1, En1, Ptx3, or TH gene was detected from a group of cells in which Lmx1a gene expression was detected, proliferating progenitor cells before cell cycle exit can be selectively detected or selected from among the progenitor cells.

The steps above were explained in the order of (a) detection of Lmx1a gene expression and (b) detection of expression of marker genes such as Lmx1b; however, the present methods

are not restricted to this order. Detection of Lmx1a gene expression and detection of the expression of marker genes such as Lmx1b may be performed simultaneously using different tags and such. Alternatively, detection of Lmx1a gene expression may be performed after detecting the expression of marker genes such as Lmx1b. In this case, proliferating progenitor cells can be selected by selecting the group expressing the Lmx1a gene from among the group of cells in which the expression of marker genes such as Lmx1b was not detected.

"Proliferating dopaminergic neuron progenitor cells before cell cycle exit", "dopaminergic neuron progenitor cells after cell cycle exit", and "mature dopaminergic neurons" can be separately detected or selected by further adding to the above method, which detects or selects progenitor cells that have exited the cell cycle and those that have not, the step of detecting the expression of the DAT gene and/or ADH2 gene, which are marker(s) that detect the above cells which have differentiated into mature dopaminergic neurons.

For example, Lmx1a gene expression is first detected using the aforementioned probe, antibody, or such and dopaminergic neurons and progenitor cells are selected. Next, in the group of cells which was selected using Lmx1a gene expression as an index, the expression of the DAT gene or ADH2 gene is examined using their respective probe, antibody, or such. Cells in which the expression of the DAT gene or ADH2 gene was detected at this point are detected or selected as mature dopaminergic neurons. On the other hand, the group of cells in which the expression of the DAT gene or ADH2 gene was not detected is further examined for expression of marker genes such as Lmx1b. Cells in which expression of marker genes such as Lmx1b was detected at this point can be identified as postmitotic precursor cells and cells in which the expression of marker genes such as Lmx1b was not detected can be identified as proliferating progenitor cells before cell cycle exit. The methods of the present invention for selecting or detecting dopaminergic neurons or progenitor cells thereof are not restricted to the orders of detection shown herein, and the order of the detection steps can be suitably determined.

Also, the conditions for designing probes and primers, the stringency conditions, and the definition of hybridization used in the methods of the present invention for detecting or selecting dopaminergic neurons or progenitor cells thereof are the same as the conditions and such used for the probes and primers for detecting Lmx1a gene expression. The methods for producing the antibodies, the types of antibodies, and such used in the methods of the present invention for detecting or selecting dopaminergic neurons or progenitor cells thereof are the same as for the anti-Lmx1a antibodies described above.

Moreover, the present invention provides kits for distinguishing dopaminergic neurons and/or progenitor cells thereof. As mentioned above, by combining an Lmx1a marker polynucleotide or an anti-Lmx1a antibody of the present invention with a marker polynucleotide probe or antibody against one or more genes selected from the group consisting of Lmx1b, Nurr1,

En1, Ptx3, TH, DAT, and ADH2, it becomes possible to selectively detect and select progenitor cells or to detect and select a group of cells that have exited the cell cycle and of those that have not from among progenitor cells. Therefore, sets of reagents that combine an above Lmx1a marker polynucleotide or anti-Lmx1a antibody with a marker polynucleotide probe or antibody against one or more genes selected from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT and ADH2, are useful as kits for distinguishing dopaminergic neurons and/or progenitor cells thereof. For example, by selecting one or more of Lmx1b, Nurr1, En1, Ptx3 or TH from the group consisting of Lmx1b, Nurr1, En1, Ptx3, TH, DAT and ADH2, and combining a marker polynucleotide probe or antibody against that selected gene with an Lmx1a marker polynucleotide or anti-Lmx1a antibody, the kits become effective in detecting/selecting progenitor cells that have exited the cell cycle and those that have not from among the progenitor cells. Alternatively, by selecting DAT or ADH2 and combining its marker polynucleotide probe or antibody with the Lmx1a marker polynucleotide or the anti-Lmx1a antibody, the kits become effective in detecting/selecting cells by distinguishing between progenitor cells and mature neurons.

<Screening for Differentiation-inducing reagents for dopaminergic neurons>

Since Lmx1a is a marker specifically expressed in dopaminergic neurons from a relatively early stage of development, it can also be used to screen for reagents that induce the differentiation of dopaminergic neurons. Specifically, the ability of a test substance to induce dopaminergic neuron differentiation can be determined by letting the test substance act on a suitable cellular sample and detecting Lmx1a expression. Therefore, the present invention provides methods of screening for candidate compounds of dopaminergic neuron differentiation-inducing reagents by using Lmx1a expression as an index. Lmx1a expression can be detected by using either a marker polynucleotide probe or an anti-Lmx1a antibody of the present invention.

The cellular samples used herein are preferably cellular samples comprising cells of the ventral midbrain region or cells that can be induced to differentiate into dopaminergic neurons, such as ES cells with pluripotency. Known methods for inducing the differentiation of dopaminergic neurons *in vitro* comprise methods which use, as the starting material, known ES cells, bone marrow interstitial cells, nerve-derived immortalized cell lines (Japanese Patent Kohyo Publication No. (JP-A) H08-509215 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication); JP-A H11-506930; JP-A 2002-522070), primordial neuron cells (JP-A H11-509729), etc. Moreover, methods for inducing dopaminergic cells from neural tissues that do not normally produce dopamine, such as the striatum and cortex, are also known (JP-A H10-509319). Thus, these cells are preferably used

as the cellular samples on which the candidate compounds for dopaminergic neuron differentiation-inducing reagents act.

Herein, any compound may be used as a test substance to be contacted with the cells. Examples comprise gene library expression products, synthetic low-molecular weight compound libraries, synthetic peptide libraries, antibodies, substances released by bacteria, cells (microbial, plant, or animal) extracts, cell culture supernatants (microbial, plant, or animal), purified or partially purified polypeptides, marine organisms, extracts from plants, animals, and such, soil, and random phage peptide display libraries. Test substances screened by the present methods show the ability to induce dopaminergic neuron differentiation. Therefore, such substances can become candidate therapeutic drugs for diseases caused by some defect in dopaminergic neurons, and can be considered effective.

Cell differentiation can be determined by comparing the Lmx1a expression level with that of cells which have not contacted the test substances. Alternatively, cell differentiation can also be determined by comparing the state of the cells, in addition to Lmx1a expression. For example, cell differentiation may be detected through morphological observation under a microscope, or by detecting or quantifying substances, such as dopamine, that are produced in the cells.

<Analysis of Lmx1a Expression Region>

An expression regulatory region of Lmx1a can be cloned from genomic DNA by known methods using a sequence of an Lmx1a gene. For example, methods for establishing the transcriptional start site, such as S1 mapping, are known and can be used (Cell Engineering, Supplement 8, New Cell Engineering Experiment Protocol, Cancer Research Division, The Institute of Medical Science, The University of Tokyo ed., Shujunsha Publishing (1993) pp. 362-374). In general, the expression regulatory region of a gene can be cloned by screening a genomic DNA library, using a probe DNA comprising a 15-100 bp segment, and preferably a 30-50 bp segment, of the 5' terminus of the gene. A clone obtained in this manner contains a 5' non-coding region of 10 kbp or more, and is shortened or fragmented by exonuclease treatment, or such. Finally, the shortened sequence portion, comprising a potential expression regulatory region, is evaluated using a reporter gene to determine the strength, regulation, and such, of its expression, thereby making it possible to determine the minimum unit required to maintain the activity of the Lmx1a expression regulatory region.

Gene expression regulatory regions can be predicted using a program such as Neural Network (http://www.fruitfly.org./seq_tools/promoter.html; Reese *et al.*, Biocomputing: Proceedings of the 1996 Pacific Symposium, Hunter and Klein ed., World Scientific Publishing Co., Singapore, (1996)). Programs for predicting the minimum unit required for the activity of

an expression regulatory region are also known and can be used (http://biosci.cbs.umn.edu./software/proscan/promoterscan.htm; Prestridge (1995) J. Mol. Biol. 249: 923-932).

The expression region of the Lmx1a gene isolated in this way can be used to produce desired polypeptides/proteins *in vivo* in a dopaminergic neuron-specific manner at all developmental stages of the dopaminergic neurons. Also, since Lmx1a is a marker specifically expressed in dopaminergic neurons from a relatively early stage of development, it can also be used to screen dopaminergic neuron differentiation-inducing reagents. Specifically, a vector is first prepared in which a reporter gene which can be detected is introduced under the regulation of the expression region of the Lmx1a gene, and suitable cells are transformed with this vector. Then, a test substance is contacted with these cells and induction of reporter gene expression by this test substance is detected. When expression of the reporter gene is detected, the test substance is judged to induce the differentiation of dopaminergic neurons.

<Lmx1a-Binding Proteins>

A sequence of 60 similar amino acid residues is conserved among proteins encoded by homeobox genes. This conserved region is called the "homeodomain" and the DNA region encoding the homeodomain is called the "homeobox". The homeodomain forms a DNA-binding domain with a helix-turn-helix conformation and binds to specific nucleotide sequences by entering the space between the two strands of the DNA. Homeobox gene products are thus thought to function as transcription factors that activate or inactivate the transcription of other genes. On the other hand, the LIM domain has a structure similar to the Zinc finger, consisting of 60 amino acid residues in which the positions of six cysteines and one histidine are conserved. Unlike the Zinc finger, DNA-binding ability has not been detected and the LIM domain is thought to be involved in protein-protein interactions. The LIM domain is thought to suppress the function of the homeodomain by intramolecular binding with the homeodomain. Further, it is thought that when an activator protein binds to the LIM domain, the homeodomain shows DNA-binding activity.

Since the Lmx1a gene is a homeobox gene having a LIM domain, it is thought to regulate the transcription of other genes by binding to activator proteins *in vivo*. Therefore, proteins that bind to Lmx1a may be used to regulate *in vivo*, *ex vivo* and *in vitro* differentiation, maturation and/or function of dopaminergic neurons. To identify Lmx1a binding proteins, Lmx1a is first contacted with a candidate protein, and the presence or absence of binding is detected. In this step, Lmx1a can be immobilized onto a support. The candidate proteins are not especially restricted, and comprise, for example, gene library expression products, natural substances derived from marine organisms, extracts of various cell types, known compounds and

peptides, natural substances derived from plants, body tissue extracts, microbial culture supernatants, and peptide groups randomly produced by the phage display method and such (J. Mol. Biol. 222: 301-10 (1991)). However, when searching for proteins that actually interact with Lmx1a, proteins expressed in dopaminergic neurons are particularly preferably selected as the candidate proteins. Also, candidate proteins may be labeled for ease of binding detection.

<Inhibition of Lmx1a Expression>

The present invention revealed that Lmx1a is expressed in dopaminergic neurons at all stages of differentiation, from proliferating dopaminergic neuron progenitor cells to cells after cell cycle exit. As a result, Lmx1a was considered to be involved in *in vivo* differentiation, maturation and/or function of dopaminergic neurons. Therefore, substances that inhibit the expression of the Lmx1a gene may be utilized to control *in vivo*, *ex vivo*, and *in vitro* differentiation, maturation and/or function of dopaminergic neurons. Examples of substances capable of inhibiting gene expression include antisense nucleic acids, ribozymes, and double-stranded RNAs (small interfering RNAs; siRNAs). Thus, the present invention provides such antisense nucleic acids, ribozymes, and double-stranded RNAs.

Examples of antisense mechanisms that suppress target gene expression include: (1) the inhibition of transcription initiation via triplex formation, (2) transcription suppression through hybrid formation at sites of local open-loop structures formed by RNA polymerase, (3) transcription inhibition through hybrid formation with RNA during synthesis, (4) splicing suppression through hybrid formation at intron-exon junctions, (5) splicing suppression through hybrid formation at sites of spliceosome formation, (6) suppression of mRNA migration to the cytoplasm through hybrid formation with mRNA, (7) splicing suppression through hybrid formation at a capping site or poly A addition site, (8) suppression of translation initiation through hybrid formation at binding sites of translation initiation factors, (9) translation suppression through hybrid formation at ribosome binding sites, (10) suppression of peptide chain elongation through hybrid formation at mRNA coding regions or polysome binding sites, and (11) suppression of gene expression through hybrid formation at sites of nucleic acid/protein interaction (Hirashima and Inoue, "New Biochemistry Experiment Course 2, Nucleic Acids IV, Gene Replication and Expression", Japanese Biochemical Society edit., Tokyo Kagaku Dozin Publishing, pp. 319-347 (1993)).

The Lmx1a antisense nucleic acids of the present invention may be nucleic acids that inhibit gene expression by any of the mechanisms described in (1) to (11) above. Namely, they may contain an antisense sequence not only to a sequence of a coding region, but also to a sequence of a non-coding region of a target gene whose expression is to be inhibited. A DNA that encodes an antisense nucleic acid can be used by linking it to a suitable regulatory sequence

that allows its expression. The antisense nucleic acids do not need to be completely complementary to a coding region or non-coding region of a target gene, as long as they can effectively inhibit expression of this gene. Such antisense nucleic acids have a chain length of at least 15 bp or more, preferably 100 bp or more, and more preferably 500 bp or more, and are normally within 3000 bp, preferably within 2000 bp, and more preferably within 1000 bp. It is preferable that such antisense nucleic acids share an identity of 90% or more, and more preferably 95% or more, with the complementary chain of a target gene transcription product. These antisense nucleic acids can be prepared according to the phosphorothionate method (Stein (1988) Nucleic Acids Res. 16: 3209-21) or the like, using the sequence information of the Lmx1a gene.

"Ribozyme" is a generic term referring to catalysts with an RNA component, and ribozymes are broadly classified into large ribozymes and small ribozymes. Large ribozymes cleave the phosphate-ester bonds of a nucleic acid, and after reaction, they leave a 5'-phosphoric acid and 3'-hydroxyl group at reaction sites. Large ribozymes are further classified into (1) group I intron RNAs, which carry out guanosine-initiated trans-esterification reactions at 5'-splice sites, (2) group II intron RNAs, which perform two-step self-splicing reactions via a lariat structure, and (3) RNA components of ribonuclease P, which cleave precursor tRNAs at their 5' side via hydrolysis reactions. In contrast, small ribozymes are comparatively small structural units (about 40 bp) that cleave RNAs, forming 5'-hydroxyl groups and 2'-3' cyclic phosphoric acids. Small ribozymes include, for example, hammerhead-type ribozymes (Koizumi *et al.* (1988) FEBS Lett. 228: 225) and hairpin-type ribozymes (Buzayan (1986) Nature 323: 349; Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112). Since ribozymes are easily altered and synthesized, various modification methods are known. For example, hammerhead-type ribozymes that recognize and cleave nucleotide sequence UC, UU, or UA within a target RNA can be created by designing the substrate binding portion of a ribozyme to be complementary to an RNA sequence near the target site (Koizumi *et al.* (1988) FEBS Lett. 228: 225; M. Koizumi and E. Ohtsuka (1990) Protein, Nucleic Acid and Enzyme 35: 2191; Koizumi *et al.* (1989) Nucleic Acids Res. 17: 7059). Hairpin-type ribozymes can also be designed and produced using known methods (Kikuchi and Sasaki (1992) Nucleic Acids Res. 19: 6751; Kikuchi (1992) Chemistry and Biology 30: 112).

The antisense nucleic acids and ribozymes of the present invention can also be used in viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses, and such, or non-viral vectors that use liposomes, or naked DNAs, to control gene expression in cells using *ex vivo* or *in vivo* gene therapy.

In 1998, a phenomenon was observed in nematodes in which RNAs interfered with each other, causing them to lose function (RNA interference) (Fire *et al.* (1998) Nature 391: 806-11).

RNA interference is a phenomenon in which RNAs with the same nucleotide sequence are degraded when an artificial double-stranded RNA is introduced into cells. Subsequent research suggests that RNA silencing phenomena such as RNA interference are cellular mechanisms for eliminating defective mRNA and defending the cells against transposons, viruses, and other parasites. At present, double-stranded RNAs (small interfering RNAs; siRNAs) are used as tools for suppressing the expression of numerous genes, and methods for treating and preventing diseases by using siRNA to suppress the expression of genes that cause diseases are being studied. There are no particular limitations as to the siRNAs of the present invention, provided that they inhibit the transcription of Lmx1a mRNA. Normally, the siRNAs are a combination of a sense chain and an antisense chain to a target mRNA sequence, and they normally have a nucleotide length from at least ten to the same number of nucleotides as the target mRNA. These siRNAs are preferably 15 to 75 nucleotides long, preferably 18 to 50, and more preferably 20 to 25 nucleotides.

Known methods can be used to introduce siRNAs into cells in order to suppress Lmx1a expression. For example, a DNA encoding in a single strand two RNA chains that compose an siRNA can be designed and then incorporated into an expression vector, cells can be transformed with the expression vector, and the siRNA can be expressed in the cells in the form of a double-stranded RNA with a hairpin structure. Plasmid expression vectors that continuously produce siRNAs by transfection have also been designed (for example, RNAi-Ready pSIREN Vector, and RNAi-Ready pSIREN-RetroQ Vector (BD Biosciences Clontech)).

The nucleotide sequence of an siRNA can be designed using a computer program such as that disclosed at the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). Kits for screening for functional siRNAs are also commercially available and can be used (for example, BD Knockout RNAi System (BD Biosciences Clontech).

All prior art documents cited herein are incorporated by reference.

Examples

Hereinbelow, the present invention will be specifically described using Examples, but it is not to be construed as being limited thereto.

[Example 1] Isolation and Sequence Analysis of a Gene Specific to Dopaminergic Neuron Progenitor Cells

To isolate a gene specific to dopaminergic neuron progenitor cells, the midbrain ventral region of E12.5 mice was cut into two regions in the dorsoventral direction, and genes specifically expressed in the most ventral region containing dopaminergic neurons were

identified by the subtraction (N-RDA) method. One of the isolated cDNA fragments was a fragment encoding Lmx1a. Lmx1a encodes a protein comprising a LIM domain and a homeodomain.

1 N-RDA method

1-1. Adapter preparation

The following oligonucleotides were annealed to each other, and prepared at 100 μM: (ad2: ad2S+ad2A, ad3: ad3S+ad3A, ad4: ad4S+ad4A, ad5: ad5S+ad5A, ad13: ad13S+ad13A)

ad2S: cagctccacaacctacatcattccgt (SEQ ID NO: 1)

ad2A: acggaatgatgt (SEQ ID NO: 2)

ad3S: gtccatcttctctctgagactctggt (SEQ ID NO: 3)

ad3A: accagagtctca (SEQ ID NO: 4)

ad4S: ctgatgggtgtcttctgtgagtgtgt (SEQ ID NO: 5)

ad4A: acacactcacag (SEQ ID NO: 6)

ad5S: ccagcatcgagaatcagtgtgacagt (SEQ ID NO: 7)

ad5A: actgtcacactg (SEQ ID NO: 8)

ad13S: gtcgatgaacttcgactgtcgatcgt (SEQ ID NO: 9)

ad13A: acgatcgacagt (SEQ ID NO: 10).

1-2. cDNA synthesis

Ventral midbrain regions were cut out of E12.5 mouse embryos (Japan SLC), and divided into two sections in the dorsoventral direction. Total RNA was prepared using an RNeasy Mini Kit (Qiagen), and double-stranded cDNA was synthesized using a cDNA Synthesis Kit (Takara). After digestion with restriction enzyme RsaI, ad2 was added. ad2S was used as the primer to amplify the cDNA using 15 PCR cycles. The conditions for amplification were: a 5-minute incubation at 72°C; 15 reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C; and finally a two-minute incubation at 72°C. In all cases, N-RDA PCR was carried out using a reaction solution containing the following components.

10x ExTaq	5 μl
2.5 mM dNTP	4 μl
ExTaq	0.25 μl
100 μM primer	0.5 μl
cDNA	2 μl
Distilled water	38.25 μl

1-3. Driver production

The ad2S-amplified cDNA was further amplified by five PCR cycles. The conditions for amplification were: incubation at 94°C for two minutes; five reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C; and finally a two-minute incubation at 72°C. The cDNA was purified using a Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. 3 μg was used for each round of subtraction.

1-4. Tester production

The ad2S amplified cDNA was further amplified by five PCR cycles. The conditions for amplification were: incubation at 94°C for two minutes; five reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C; and a final two-minute incubation at 72°C. The cDNA was purified using a Qiaquick PCR Purification Kit (Qiagen), and digested with RsaI. ad3 was added to 60 ng of the RsaI-digested cDNA.

1-5. First round of subtraction

The tester and driver produced in Sections 1-3 and 1-4 above were mixed, ethanol precipitated, and then dissolved in 1 μl of 1x PCR buffer. After a five-minute incubation at 98°C, 1 μl of 1x PCR buffer + 1M NaCl was added. After another five-minute incubation at 98°C, the tester and driver were hybridized at 68°C for 16 hours.

With ad3S as the primer, the hybridized cDNA was amplified by ten cycles of DNA (incubation at 72°C for five minutes; then ten reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C. Next, the amplified cDNA was digested with Mung Bean Nuclease (Takara) and purified using a Qiaquick PCR Purification Kit. Then, it was amplified by 13 PCR cycles. The conditions for amplification were: incubation at 94°C for two minutes; 13 reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C; and a final two-minute incubation at 72°C.

1-6. Normalization

1 μl of 2x PCR buffer was added to 8 ng of the cDNA amplified in the first round of subtraction. After incubating at 98°C for five minutes, 2 μl of 1x PCR buffer + 1 M NaCl was added. After another five minutes of incubation at 98°C, the cDNA was hybridized at 68°C for 16 hours.

The hybridized cDNA was digested with RsaI and then purified using a Qiaquick PCR Purification Kit. This was then amplified by eleven PCR cycles using ad3S as the primer (incubation at 94°C for two minutes; then eleven reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and two minutes at 72°C; and finally a two-minute incubation at 72°C). The PCR product was then digested with RsaI and ad4 was then added.

1-7. Second Round of Subtraction

20 ng of the cDNA to which ad4 was added in Section 1-6 above was used as the tester and mixed with the driver of 1-3 above. The same subtraction procedure as used in Section 1-5 above was performed. Finally, ad5 was added to the cDNA following RsaI digestion.

1-8. Third Round of Subtraction

2 ng of the cDNA to which ad5 was added in Section 1-7 above was used as the tester and mixed with the driver of 1-3 above. The same subtraction procedure as used in section 1-5 above was performed. Finally, ad13 was added to the cDNA following RsaI digestion.

1-9. Fourth Round of Subtraction

2 ng of the cDNA to which ad13 was added in Section 1-8 above was used as the tester and mixed with the driver of 1-3 above. The same subtraction procedure as used in Section 1-5 above was performed. The amplified cDNA was cloned into pCRII vector (Invitrogen) and its nucleotide sequence was analyzed using the ABI3100 sequence analyzer.

[Example 2] Expression Analysis of Lmx1a

1. To confirm the expression of Lmx1a in dopaminergic neurons, expression analyses of Lmx1a, Lmx1b, Nurr1, and tyrosine hydroxylase (TH) mRNAs by *in situ* hybridization were carried out according to the following protocol: Nurr1 and TH are markers whose expression is known to be induced for the first time in dopaminergic neuron progenitor cells after cell cycle exit. Lmx1b is a transcription factor marker known to be expressed at an extremely low level at the stage of proliferating progenitor cells, but its expression starts to increase to a high level after cell cycle exit.

First, E12.5 mouse embryos were extracted, fixed in 4% PFA/PBS(-) at 4°C for two hours, exchanged with 20% sucrose/PBS(-) at 4°C overnight, and embedded in O.C.T. Sections of 12 μm thickness were prepared and after drying on a slide glass, the sections were fixed again in 4% PFA at room temperature for 30 minutes. After washing with PBS, hybridization was carried out at 68°C for 40 hours (1 μg/ml DIG-labeled RNA probe, 50% formamide, 5x SSC, 1% SDS, 50 μg/ml yeast RNA, 50 μg/ml Heparin). The sections were then washed at 68°C (50% formamide, 5x SSC, 1% SDS) and treated with RNase (0.05 μg/ml RNase) at room temperature for five minutes. After washing with 0.2x SSC at 68°C and washing with 1x TBST at room temperature, blocking was carried out (Blocking reagent: Roche). The sections were then reacted overnight at 4°C with alkaline phosphatase-labeled anti-DIG antibody (DAKO), washed (1x TBST, 2 mM Levamisole), and color developed using NBT/BCIP (DAKO) as the substrate.

Expression analyses by *in situ* hybridization revealed that Lmx1a was expressed in the most ventral region of the midbrain, similarly to TH, Lmx1b, and Nurr1, at the E12.5 stage, which corresponds to the period of dopaminergic neuron development (Fig. 2).

2. Next, Lmx1a protein expression was confirmed using Lmx1a-specific antibodies. Furthermore, double staining with TH and En1, which are markers of dopaminergic neuron progenitor cells after cell cycle exit, was also conducted. En1 is a marker whose expression is induced for the first time in dopaminergic neuron progenitor cells after cell cycle exit.

For the anti-Lmx1a polyclonal antibodies, *E. coli* (JM 109 strain) were first transformed with a vector in which GST and a DNA region corresponding to amino acids 271 to 307 of Lmx1a were fused, then expression was induced by IPTG and the antigens necessary for immunization were recovered. After recovery, rabbits were immunized, their blood was collected, and antibodies were obtained from the serum by affinity purification using the GST-Lmx1a antigens that were used for immunization.

E12.5 mouse embryos were extracted, fixed in 4% PFA/PBS(-) at 4°C for two hours, exchanged with 20% sucrose/PBS(-) at 4°C overnight, and embedded in O.C.T. Sections of 12 μm thickness were prepared, fixed on a slide glass and dried at room temperature for 30 minutes, then infiltrated again with PBS(-). Then, blocking (Block Ace) was performed at room temperature for 30 minutes, primary antibodies were reacted at room temperature for one hour, and further reacted at 4°C overnight. Washing with 0.1% Tween-20/PBS(-) at room temperature for 15 minutes was performed three times. Next, samples were reacted with fluorescent-labeled secondary antibodies at room temperature for one hour, washed in the same way, then washed with PBS(-) at room temperature for ten minutes, and embedded.

As a result, expression patterns similar to those detected by *in situ* hybridization were observed (Figs. 3 and 4). Not only the mRNAs but also the Lmx1a proteins were revealed to be expressed in the E12.5 midbrain. Double staining with TH and En1 confirmed co-expression of Lmx1a and these proteins in the same cell, and their expression regions were also revealed to be completely identical in the dorsoventral direction (Figs. 3 and 4). In contrast with TH and En1, Lmx1a expression was also detected in the ventricular side. This region (Ventricular Zone (VZ)) comprises proliferating progenitor cells that will eventually differentiate into dopamine neurons.

3. Therefore, to confirm that Lmx1a is expressed in proliferating progenitor cells, bromodeoxyuridine (BrdU) incorporation and Lmx1a expression in E12.5 mouse midbrain were detected by immunostaining.

BrdU (Sigma) was injected (10 mg/ml in 0.9% saline injected to give 50 μg/g body

weight) into the abdominal cavity of pregnant mice two hours before extracting E12.5 mouse embryos, and BrdU was incorporated into the DNA of proliferating cells. Sections were prepared in the same way as in conventional immunostaining. Lmx1a proteins were first detected using the anti-Lmx1a antibody, then sections were fixed again (2% PFA, room temperature, 30 minutes), treated with hydrochloric acid (2N HCl, 37°C, 30 minutes), and BrdU was detected using anti-BrdU antibody.

The results revealed that BrdU was incorporated in many Lmx1a-positive cells of the VZ region (Fig. 5).

4. Lmx1a expression in dopaminergic neurons of mice after birth was also examined. Lmx1a expression was detected by the method of 1, described above, except that the midbrain sections of 7-day-old (P7) mice were used instead of those of E12.5 mouse embryos, and the dopaminergic neuron marker DAT was used as the marker for comparison. DAT is a marker known to be expressed only once dopaminergic neurons have advanced into maturation.

The results revealed that Lmx1a is expressed in regions where DAT is expressed (Fig. 6).

5. Furthermore, to examine whether Lmx1a is also specifically expressed in dopaminergic neurons in humans, RT-PCR was performed using RNA from the adult brain region.

cDNA synthesis was performed for1 μg of total RNA from various regions of the human brain purchased from Clontech using RNA PCR kits (TaKaRa). Then, using cDNA equivalent to 10 ng, 1 ng, and 0.1 ng as a template, PCR was performed in the reaction system below.

10x ExTaq	2 μl
2.5 mM dNTP	1.6 μl
ExTaq	0.1 μl
100 μM primers	0.2 μl each
cDNA	1 μl
Distilled water	14.9 μl

The conditions for amplication were: a two-minute incubation at 94°C; 37 reaction cycles of 30 seconds at 94°C, 30 seconds at 65°C and 30 seconds at 72°C; and a final two-minute incubation at 72°C.

The primer sequences used were as follows:

Human Lmx1a: TGAAGAAAGTCTCTGCAAGTCAGCCC (SEQ ID NO: 11) / CACCACCGTTTGTCTGAGCAGAGCTC (SEQ ID NO: 12).

The results revealed that in humans also, Lmx1a is expressed in the midbrain substantia nigra region, where dopaminergic neurons are present (Fig. 7). Further, expression similar to

that of mice was also shown in other brain regions, such as the hippocampus, and the expression was also revealed to be maintained in the dopaminergic neurons of adults.

Based on the above results, the timing of expression of Lmx1a in dopaminergic neurons was compared to that of other dopaminergic neuron markers (Fig. 8). The expression of Lmx1a is maintained from the stages of proliferating progenitor cells to cells after cell cycle exit and even in adults. On the other hand, expression of the known Nurr1, En1, Ptx3, and TH is induced for the first time after cell cycle exit and the expression of DAT and ADH2 starts only after this expression has progressed. Lmx1b expression can also be observed in proliferating progenitor cells; however, the expression level is extremely low and increases to a high level after cell cycle exit. As such, the Lmx1a expression pattern is different from known markers and Lmx1a becomes a useful marker in detecting dopaminergic neurons.

Industrial Applicability

The expression of Lmx1a was revealed to be maintained in dopaminergic neurons from the stages of proliferating progenitor cells to cells after cell cycle exit and even in adults. On the other hand, the expression of known dopaminergic neuron markers such as Nurr1, En1, Ptx3, and TH is induced for the first time after cell cycle exit, and the expression of DAT and ADH2 starts only after this expression has progressed. Lmx1b expression can also be detected in proliferating progenitor cells; however, the expression level is extremely low and increases to a high level after cell cycle exit. The Lmx1a expression pattern is different from that of known markers and Lmx1a is specifically expressed in dopaminergic neurons at all differentiation stages, from proliferating dopaminergic neuron progenitor cells to cells after cell cycle exit. Thus Lmx1a is considered to be a useful marker in detecting dopaminergic neurons (see Fig. 8).

Lmx1a is a marker expressed at an earlier stage than conventional markers. Therefore, by using the expression of Lmx1a in cells as an index, it becomes possible to select cells suitable in terms of safety, survival rate, and network formation ability for use in transplantation therapy for neurodegenerative diseases such as Parkinson's disease. Furthermore, since Lmx1a is a marker expressed at an earlier stage, it is expected to be especially effective when screening for differentiation-inducing reagents for dopaminergic neurons. Such genes, widely expressed throughout the differentiation stages from proliferating dopaminergic neuron progenitor cells before cell cycle exit (progenitor cells at the initial stage of neuron formation) to mature cells, are considered to be useful in revealing the various factors involved in the maturation process of neurons, and the various factors involved in the expression of neuron function. Moreover, elucidation of such factors is expected to greatly contribute to the therapy of neurodegenerative diseases.